bio-protocol Preprint

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Plasmids and lentivirus production

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An abbreviated version of this protocol was published in eLIFE in Apr 2022

Multi-omic rejuvenation of human cells by maturation phase transient reprogramming

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Detailed protocol

Plasmid cloning

To clone the FUW-tetO-GFP-hOKMS plasmid, the GFP-IRES sequence was amplified using the following forward and reverse primers, which also introduced restriction sites (bold): AAG**GGATCC**GCCACCATGGTGAGCAAGGGCG-AGGAGCT and AA**ACCGGT**TTATCATCGTGTTTTTCAAA. PCR amplification was carried out in 20 ml of Phusion HF buffer (1x) containing 0.02 U/ml Phusion DNA polymerase (Thermo Scientific), 0.5 mM forward primer, 0.5 mM reverse primer and 0.2 mM dNTPs with the following PCR program:

- 98°C for 30 seconds
- 35 cycles of:
  - 98°C for 10 seconds
  - 70°C for 30 seconds
  - 72°C for 1 minute
- 72°C for 10 minutes
- 4°C hold

Following amplification, the products were run on an agarose gel to confirm that the product was of the correct size (1500 bp). This PCR product was subsequently purified with the Monarch PCR purification kit (New England Biolabs/NEB). The GFP-IRES sequence and FUW-tetO-hOKMS plasmid (Addgene 51543, a gift from Cacchiarelli et al., 2015) were simultaneously digested with BamHI-HF (NEB) and AgeI-HF (NEB) to generate linear fragments with compatible overhangs. Enzymatic digestion was carried out in 50 ml of Cutsmart buffer (1X) containing 1 mg of DNA, 0.4 U/ml of BamHI-HF and 0.4 U/ml AgeI-HF at 37°C for 15 minutes. Following digestion, the reaction was inactivated by incubating at 65°C for 20 minutes. The resulting fragments were purified with the Monarch PCR purification kit (NEB) and ligated together with T4 DNA ligase (NEB). DNA ligation was carried out in 40 ml of T4 DNA ligase buffer containing 157.04 ng of GFP-IRES, 299.93 ng of FUW-tetO-hOKMS, and 150 U/ml of T4 DNA ligase at 14°C overnight. The ligation reaction was inactivated by incubating at 65°C for 10 minutes and used to transform DH5α library efficient bacteria (Invitrogen). Transformed bacteria were grown on LB plates containing 100 µg/ml ampicillin. Colonies were selected and expanded. Following this, DNA was extracted from these expanded cultures using the QIAprep Spin Miniprep Kit (Qiagen) and validated by sanger sequencing performed by GENEWIZ.

Generating lentiviruses

HEK293T cells were grown in HEK293T medium (DMEM, 10% FBS, 1X Glutamax and 1X Pen/Strep). Upon reaching 90% confluency, the cells were passaged using Trypsin-EDTA (0.05%) and were seeded onto 10 cm dishes at a density of 4 million cells per dish so that they would reach 90% confluency the subsequent day. The following day, the transfection reagent was prepared by adding 3.5 mg of the pMD2.g plasmid, 6.5 mg of the psPAX2 plasmid and 10 mg of either the FUW-M2rtTA plasmid (Addgene 20342, a gift from Hockemeyer et al., 2008) or the FUW-tetO-GFP-hOKMS plasmid to 1 ml of Opti-MEM Reduced-Serum Medium. 60 ml of TransIT-293 transfection reagent was added dropwise to this mixture. The final mixture was briefly vortexed and incubated at room temperature for 30 minutes to allow transfection complexes to form. The transfection reagent was added to the HEK293T cells after changing the HEK293T media. The media containing lentiviruses was collected 48 hours after the transfection and filtered through a 0.45 mm polyethersulfone filter to remove cellular debris. The lentiviruses were stored in 3 ml aliquots at -80°C until needed.

GFP-IRES sequence

GGATCCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCA